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The Kruppel-like Zinc Finger Protein ZNF224 Recruits the Arginine Methyltransferase PRMT5 on the Transcriptional Repressor Complex of the Aldolase A Gene*[S]

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Gene transcription in eukaryotes is modulated by the coordinated recruitment of specific transcription factors and chromatin-modulating proteins. Indeed, gene activation and/or repression is/are regulated by histone methylation status at specific arginine or lysine residues. In this work, by co-immunoprecipitation experiments, we demonstrate that PRMT5, a type II protein arginine methyltransferase that monomethylates and symmetrically dimethylates arginine residues, is physically associated with the Kruppel-like associated box-zinc finger protein ZNF224, the aldolase A gene repressor. Moreover, chromatin immunoprecipitation assays show that PRMT5 is recruited to the L-type aldolase A promoter and that methylation of the nucleosomes that surround the L-type promoter region occurs *in vivo* on the arginine 3 of histone H4. Consistent with its association to the ZNF224 repressor complex, the decrease of PRMT5 expression produced by RNA interference positively affects L-type aldolase A promoter transcription. Finally, the alternating occupancy of the L-type aldolase A promoter by the ZNF224-PRMT5 repression complex in proliferating and growth-arrested cells suggests that these regulatory proteins play a significant role during the cell cycle modulation of human aldolase A gene expression. Our data represent the first experimental evidence that protein arginine methylation plays a role in ZNF224-mediated transcriptional repression and provide novel insight into the chromatin modifications required for repression of gene transcription by Kruppel-like associated box-zinc finger proteins.

Gene transcription is controlled by the interplay of several transacting factors and chromatin-modifying activities that are sequentially recruited to the promoter region. Post-translational modifications of histone and non-histone chromosomal proteins are considered to be additional mechanisms that contribute to the epigenetic inheritance of phenotypic alterations. The temporal and combinatorial recruitment of the histone-modifying activities can determine differential outcomes in gene expression (1–3).

Histone methylation, which usually occurs on arginine or lysine residues, is involved in regulation of chromatin structure, which in turn either stimulates or inhibits gene transcription. In fact, methylation of Lys-4 and Arg-17 of histone H3 and Arg-3 of histone H4 has been associated with transcriptional activation, whereas methylation of Lys-9 and Lys-27 of histone H3 has been related to gene repression (4, 5). Arginine methylation of nucleosomal histones is catalyzed by a homogenous class of enzymes that are known as "protein arginine methyltransferases" (PRMTs).[4] In this reaction the methyl group, which is provided by the *S*-adenosyl-L-methionine, is transferred to one of the guanidinium nitrogens of arginine residues. PRMTs are divided into type I PRMTs, which catalyze monomethylation and asymmetric dimethylation of arginine residues, and type II PRMTs, which also catalyze monomethylation and, in addition, symmetric dimethylation of arginine residues (6). PRMT5, which is a mammalian II type PRMT, was discovered through a yeast two-hybrid screening and identified as Janus kinase-interacting protein 1 (7). The methylation activity of PRMT5 is addressed not only toward the histone H2A and H4, but also toward such non-histone proteins as myelin basic proteins, Sm proteins B, B′, D1, D3, and LSm4 (8–10). PRMT5 participates in the assembly of the transcriptional repressor complex on different eukaryotic promoters (11–15).

The Kruppel-like associated box (KRAB)-zinc finger proteins (KRAB-ZFPs), a well characterized family of DNA-binding factors, represent approximately one-third of the mammalian ZFPs (16). Genome screening showed that the KRAB-containing ZFP repertoire is differentially expressed between species, which suggests that this gene family is a specific of vertebrates. Moreover, the observation that only 103 orthologs of the 423 human KRAB-containing ZFPs have been

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[4] The abbreviations used are: PRMT, protein arginine methyltransferase; AldA-NRE, aldolase A-negative regulatory element; KRAB, Kruppel-associated box; KAP-1, KRAB-associated protein 1; GST, glutathione *S*-transferase; CMV, cytomegalovirus; ChIP, chromatin immunoprecipitation; re-ChIP, chromatin re-immunoprecipitation; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; siRNA, small interference RNA.

found in the rodent genome strongly suggests that these genes arose after the divergence of the primate and rodent lineages. Therefore, the protein products of these genes might play a role in regulation of gene expression involved in speciation (17, 18). At the NH_2 terminus, KRAB-ZFPs have an evolutionarily conserved regulatory domain, KRAB, which inhibits transcription (19–21). KRAB-mediated gene silencing requires the interaction of the KRAB domain with the co-repressor protein KAP-1 (KRAB-associated protein 1) (22–24) that, in turn, coordinates the activities of large macromolecular complexes that modify chromatin structure to silence gene expression (25–28). In this way, the large family of KRAB-ZFPs may regulate transcription of target genes during cellular differentiation and development. Despite the increasing body of information about the KRAB-ZFP-mediated recruitment model, we still do not know which proteins are present in the large macromolecular complex and how they mediate the repression activity of the KRAB-ZFPs on the eukaryotic promoters.

In previous reports we described the structural and functional properties of ZNF224, a member of the KRAB-ZFP family. This system allows us to analyze *in vivo* how sequence-specific transcriptional repressors recruit, at gene-specific loci, the network of proteins that are necessary to establish localized microenvironments of heterochromatin to repress gene transcription. In proliferating cells, ZNF224 specifically binds to the negative regulatory element, AldA-NRE, located in the L-type aldolase A promoter, and acts as a transcriptional repressor. The interaction between ZNF224 and AldA-NRE is responsible for the modulation of aldolase A mRNA expression during the cell cycle (29, 30). The NH_2 terminus of ZNF224 contains a well conserved KRAB domain, including a canonical box A and a degenerated box b, whereas the C terminus contains 19 C_2H_2 zinc finger motifs (31). We recently demonstrated that KRAB A is the repression domain of ZNF224 and its transcriptional repression activity requires the physical interaction with the co-repressor KAP-1. Moreover, we found that the mechanism of the transcriptional ZNF224-mediated repression depends on histone deacetylase activity (32). Finally, we demonstrated that ZNF224 and its shorter isoform, ZNF255, differentially repress aldolase gene transcription and exhibit a distinctive subcellular localization (33).

In this study, we report an additional component of the repressor complex that is recruited by ZNF224 on the aldolase A promoter to regulate transcription. In fact, by co-immunoprecipitation and GST pulldown experiments, we demonstrate that PRMT5, a type II protein arginine methyltransferase, is physically associated with the KRAB-ZFP ZNF224, the aldolase A gene repressor, with their interaction site mapping within the NH_2 terminus region of ZNF224. Moreover, using chromatin immunoprecipitation assays, we show that PRMT5 is recruited to the L-type aldolase A promoter and that it methylates *in vivo* arginine 3 of histone H4. We also demonstrate that the knockdown of PRMT5, obtained by RNA interference assay, removes the transcriptional ZNF224-mediated repression of the L-type aldolase A promoter. In addition, the alternating occupancy of the L-type aldolase A promoter by the ZNF224-PRMT5 repression complex in proliferating and growth-arrested cells suggests that these regulatory proteins play a significant role during the cell cycle modulation of aldolase A gene expression. Moreover, we were able to show, by flow cytometric assays, global changes in the methylation status of H4R3 during the cell cycle, associated to consistent expression levels of PRMT5. These data represent the first experimental evidence that protein arginine methylation exerts a crucial function in ZNF224-mediated transcriptional repression and suggest a novel model of chromatin modification that is required for repression of gene transcription by KRAB-ZFPs.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—HEK293 cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum, 100 μg/ml penicillin, and 100 units/ml streptomycin per at 37 °C in 5% CO_2. For transfection, HEK293 cells were seeded at 1×10^6 cells/10-cm plate, and 3×FLAG-ZNF224, 3×FLAG-AF4, MycPRMT5, and MycPECE expression vectors were transiently transfected with Lipofectamine reagent (Invitrogen). To obtain stable clones expressing 3×FLAG-ZNF224, we transfected HEK293 cells with p3×FLAG-ZNF224 G418-selectable plasmid. After 24 h, the medium was supplemented with 800 μg/ml G418 (Promega, Madison, WI). The cells were grown in selective medium until colony formation. The positive clones were analyzed by Western blotting using M2 anti-FLAG antibody (Sigma-Aldrich), and one of these (CL13) was chosen for further experiments. As negative control we selected a clone (CN2) from HEK293 cells transfected with the empty p3×FLAG G418-selectable plasmid.

Plasmid Construction—DNA fragments encoding the deletion mutants M16, M13, M10, M3, and M1 were amplified from the plasmid 3×FLAG-ZNF224 with the 5′-forward primer: CCCAAGCTTATGGAGACTGTTTCAGAAGCA and the following 3′-reverse primers: TGCTCTAGATCATCAGAGAGGTGTTTCTCTGCT (for M16), TGCTCTAGATCATCATCCTGTGTGGACCTTCTGGTG (for M13), TGCTCTAGATCATCATCTTCCCACATTCCTTACAATTAT (for M10), TGCTCTAGATCATCATTTGAATGGCTTCTCCCCCGT (for M3), and TGCTCTAGATTAGTGGACTCTCTGATGAATACGAAGGGCTGAG (for M1). The amplified fragments were cloned into HindIII/XbaI sites of the eukaryotic expression plasmid p3×FLAG-CMV 7.1.

Cell Cycle Synchronization and Flow Cytometric Analysis—HEK293 cells were treated with 5 mM thymidine (Sigma) or with 100 ng/ml nocodazole (Sigma) for 20 h to arrest cells in G_0 and G_2/M phases, respectively. To arrest the cells in S phase, cells treated with 5 mM thymidine were released by adding fresh medium. Synchronized cells were stained with propidium iodide and analyzed by flow cytometry of DNA using an AacScan Cytometer Apparatus (488 nm argon laser, BD Biosciences, Mountain View, CA). Cell cycle histograms were obtained with CellFit (BD Biosciences) software on an HP Workstation Apollo OS Consort 32.

Immunoprecipitation and Western Blotting—For immunoprecipitation of 3×FLAG-ZNF224 and c-MycPRMT5 complexes, anti-FLAG-M2-agarose beads and anti-c-Myc-agarose conjugate (20 μl of beads/mg of protein, Sigma) or IgG (5 μg/mg of protein, Sigma) were added to 1 mg of cell lysates,

obtained with 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Nonidet P-40, 10% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, protease inhibitor, and incubated for 3 h at 4 °C. The immune complexes were washed twice with 50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 1 mM EDTA, pH 8.0, 10% glycerol, 0.1% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride, protease inhibitor, and twice with cell lysis buffer. The protein complexes were eluted from beads by boiling in Laemmli buffer (Bio-Rad) and subjected to 8% SDS-PAGE. Western blotting was performed using antibodies against FLAG (anti-FLAG-M2, Sigma, 1:5000 dilution), anti-Myc tag (Upstate, Lake Placid, NY), and anti-PRMT5 (Upstate). The Upstate antibodies were used at a concentration of 1 μg/ml for 2 h at room temperature. Horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences, Buckinghamshire, UK) and ECL chemiluminescent reagent (Amersham Biosciences) were used.

Chromatin Immunoprecipitation Assay—Cross-linked chromatin was prepared from cycling HEK293 cells and enriched differential populations of HEK293 cells from the cell cycle phases. Briefly, 6×10^7 cells were cross-linked with 1% of fixing solution (11% HCHO, 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 50 mM Hepes, pH 8.0) and harvested in 1.5 ml of cell lysis buffer (5 mM PIPES, pH 8.0, 85 mM KCl, 0.5% Nonidet P-40, protease inhibitors). Nuclei were collected and resuspended in 300 μl of nuclear lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 0.8% SDS, and protease inhibitors). The DNA was sheared by sonication. Sonicated DNA was diluted 3-fold in dilution buffer (10 mM Tris-HCl, pH 8.0, 0.5 mM EGTA, 1% Triton X-100, 140 mM NaCl, protease inhibitors) and precleared with protein A/G plus-agarose (Santa Cruz Biotechnology, Santa Cruz, CA) at 4 °C for 1 h. The precleared extract was divided in two aliquots; one was used for control preimmune serum and the other portion was incubated with PRMT5 (Upstate), SYM11 (Upstate), and H4 symmetric dimethyl R3 (Abcam Inc., Cambridge). Beads were collected by centrifugation and then washed five times with radioimmune precipitation assay buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0, 0.5 mM EGTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 140 mM NaCl, 1 mM phenylmethylsulfonyl fluoride), once with LiCl buffer (0.25 M LiCl, 0.5% sodium deoxycholate, 1 mM Na-EDTA, 10 mM Tris-HCl, pH 8.0) and once with TE buffer (1 mM Na-EDTA, 10 mM Tris-HCl, pH 8.0). At the end, the beads were taken up in 100 μl of TE. 1 μg of RNase was added, and cross-links were reversed at 65 °C overnight. The next day, samples were adjusted to 0.5% SDS and 0.5 mg/ml proteinase K and incubated for 3 h at 50 °C. DNA was purified by phenol-chloroform extraction. ChIP samples were then analyzed by standard PCR using specific AldA-NRE primers: AldA-NRE Fw, 5′-CCCTCTGTTCCACTGGGCAAGTGAG-3′ and AldA-NRE Rev, 5′-CCATTCCAGTTCCCAGGCCTGGGTG-3′; G3PDH primers were used to amplify a negative control site: G3PDH Fw, 5′-AGGTCATCCATGACAACTTTGG-3′ and G3PDH Rev, 5′-TTGTCATACCAGGAAATGAGCT-3′. Immunoprecipitated DNA was analyzed also by quantitative PCR using a Master Mix SYBR Green (Bio-Rad) and the following primers: AldA-NRE Fw, 5′-GATTTCCCAGGCCTCTCTGTTCC-3′ and AldA-NRE Rev, 5′-CACAGTCGCAGAGTGGGCAGC-3′; pL Fw, 5′-GCTGCTCACCACACACAAGT-3′ and pL Rev, 5′-CCTCCTAAAATAACGGAGAGTCC-3′. The -fold difference value of sample relative to the corresponding negative control was calculated with the method of Chakrabarti *et al.* (34). Chromatin re-immunoprecipitation (re-ChIP) experiments were performed by using antibody-protein-DNA complexes eluted from beads by incubation for 30 min at 37 °C in 25 μl of 10 mM dithiothreitol.

siRNA Knockdown and Real-time PCR—Three stealth siRNA duplexes, targeted to different sites in human PRMT5 mRNA, were designed by the software provided by Invitrogen. The sequences of the siRNAs are the following (sense strands, 5′ → 3′): siRNA1, GGACAAUCUGGAAUCUCAGACAUAU (target site 1039–1064); siRNA2, GGCUCCAGAGAAAGCAGACAUCAUU (target site 1363–1388); and siRNA3, GCGGCCAUGUUACAGGAGCUGAAUU (target site 404–429). We transfected 70% confluent HEK293 cells with stealth RNAs or scrambled RNA (40 pmol/24-multiwell plates) using Lipofectamine 2000 (Invitrogen) and Opti-MEM I (Invitrogen).

Fresh medium was added 6 h after transfection. Forty-eight hours after transfection, cells were collected and total RNA was isolated by TRIzol (Invitrogen). One microgram of each RNA was used for cDNA synthesis with Moloney murine leukemia virus reverse transcriptase (Invitrogen). We quantified gene expression by real-time PCR using Master Mix (Bio-Rad) and specific primers for aldolase A, PRMT5, and β-actin mRNAs, respectively. For aldolase A: Fw, 5′-TCA ACC ACA CTC CGT CCA CG-3′ and RevC, 5′-GTA GCA AGT TCC GGT GCT TC-3′; for PRMT5: Fw, 5′-CTG GAT GGA GCC CAG CAC T-3′ and Rev, 5′-GGC TCG GAC CTC ATT GTA CA-3′; for β-actin: Fw, 5′-CGA CAG GAT GCA GAA GGA GA-3′ and Rev, 5′-CGT CAT ACT CCT GCT TGC TTG CTG-3′.

Protein Production and Purification—The DNA fragment encoding for ZNF224 was amplified from the plasmid described above (31) with the following primer set: Sal1224 Fw, 5′-ACG CGT CGA CAT GAC CAC GTT CAA GGA GGC AA-3′; Not1 Rev, 5′-ATA AGA ATG CGG CCG CTG AAG ACT GAG T-3′. The DNA fragment encoding for the KRAB domain of ZNF224 was amplified with the following primer set: Sal1224 Fw, 5′-ACG CGT CGA CAT GAC CAC GTT CAA GGA GGC AA-3′; Not1KRAB Rev, 5′-ATAAGAATGCGGCCGCTCACTATCCTGAATTCCCTTC-3′. PCR products were digested with Sal1 and Not1 and cloned into pGEX-4T-3 vector (Amersham Biosciences). The GST fusion proteins were expressed from BL21 cells by induction with 1 mM isopropyl 1-thio-β-D-galactopyranoside (Promega) at 30 °C for 4 h and batch-purified with glutathione-Sepharose 4B beads (Amersham Biosciences).

In Vitro Transcription/Translation and GST Pulldown Assay—The [^{35}S]Met-labeled PRMT5 protein was obtained from 1 μg of plasmid DNA template pMyc-PRMT5 using cell-free transcription-coupled translation in rabbit reticulocyte lysate (TnT Quick Coupled Transcription/Translation Systems, Promega) according to the manufacturer's protocol. For the GST pulldown reaction, the labeled PRMT5 was added to GST-ZNF224 or to GST-KRAB or to GST alone (5 μg each), and the reactions were performed for 3 h at 4 °C in T&G buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 2.5 mM EGTA, pH 8.0, 1 mM EDTA, pH 8.0,

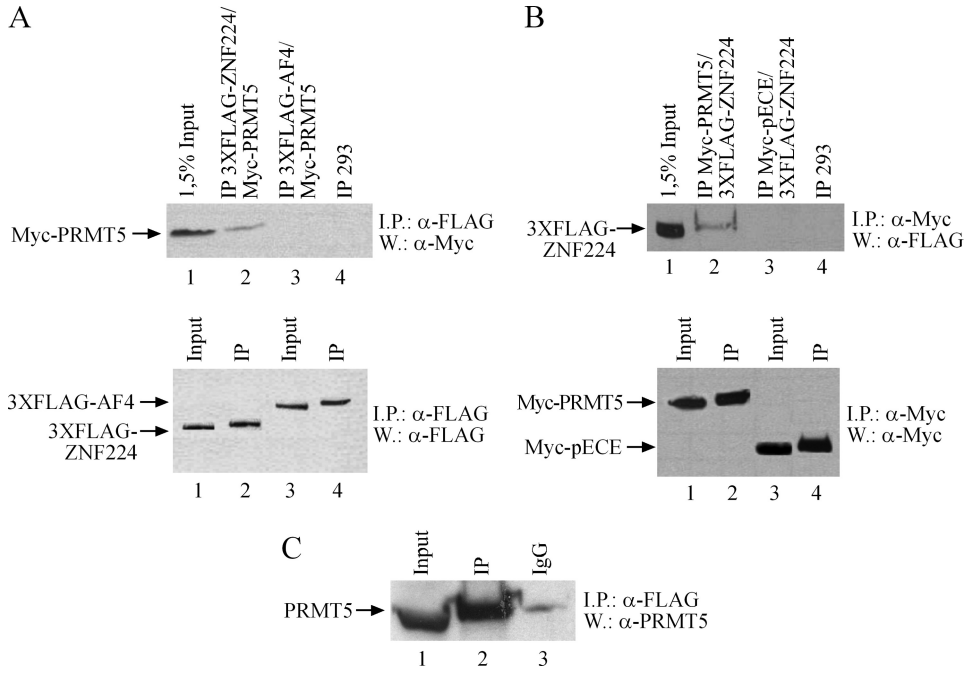

FIGURE 1. **Association of the ZNF224 protein with PRMT5.** *A*, PRMT5 and ZNF224 exogenously expressed in HEK293 cells were immunoprecipitated with anti-FLAG antibody. After immunoprecipitation, Western blot analysis was performed with the indicated antibody. Myc-PRMT5 was present in the complex of 3×FLAG-ZNF224 (*lane 2*) but not in the negative control 3×FLAG-AF4 (*lane 3*). The *lower panel* shows Western blot with anti-FLAG antibody performed on cell lysates co-transfected with 3×FLAG-ZNF224 and Myc-PRMT5 (*lane 2*) or co-transfected with 3×FLAG-AF4 and Myc-PRMT5 (*lane 4*) and immunoprecipitated with anti-FLAG antibody. The levels of input proteins are shown (*lanes 1* and *3*). *B*, PRMT5 and ZNF224 exogenously expressed in HEK293 cells were immunoprecipitated with anti-Myc antibody, followed by Western blot analysis with anti-FLAG antibody. 3×FLAG-ZNF224 interacted with Myc-PRMT5 (*lane 2*) but not with the negative control Myc-pECE (*lane 3*). The *lower panel* shows Western blot with anti-Myc antibody performed on cell lysates co-transfected with 3×FLAG-ZNF224 and Myc-PRMT5 (*lane 2*) or co-transfected with 3×FLAG-ZNF224 and Myc-PECE (*lane 4*) and immunoprecipitated with anti-Myc antibody. The levels of input proteins are shown (*lanes 1* and *3*). *C*, HEK293 cells were transfected with p3×FLAG-ZNF224, and extracts were immunoprecipitated with anti-FLAG or control immunoglobulin (IgG), as indicated. Western blot analysis was performed with antibody directed against PRMT5. Data shown were confirmed by three independent experiments.

known partner of PRMT5 (data not shown). To verify the interaction between PRMT5 and ZNF224, we co-transfected the HEK293 cells with 3×FLAG-ZNF224 and Myc-PRMT5 expression vectors, and performed a specific co-immunoprecipitation assay with a FLAG antibody followed by Western blotting with anti-c-Myc. The exogenously expressed PRMT5 clearly co-immunoprecipitated with the ZNF224 protein (Fig. 1*A*, *upper panel*, *lane 2*). To verify the specificity of the interaction between ZNF224 and PRMT5, we co-transfected HEK293 cells with Myc-PRMT5 and an unrelated FLAG-expressing vector (3×FLAG-AF4). As expected, in this condition, the immunoprecipitation assay did not reveal the presence of PRMT5 (*lane 3*), thus confirming the specificity of our experimental conditions. The *lower panel* of Fig. 1*A* shows the levels of the relative overexpressed and immunoprecipitated FLAG-tagged proteins.

To verify further the interaction of the two proteins, total extracts were prepared from cells co-transfected with 3×FLAG-ZNF224 and Myc-PRMT5 expression vectors and co-immunoprecipitated with a specific Myc antibody. The immunoprecipitated samples were analyzed by Western blotting with an anti-FLAG antibody (Fig. 1*B*). As shown in *lane 2* (*upper panel*), ZNF224 co-immunoprecipitated with Myc-PRMT5, whereas no interaction was discernable in extracts from cells transfected with unrelated Myc-pECE expression vector (*lane 3*). The overexpressed and immunoprecipitated Myc-tagged proteins levels are shown in the *lower panel* of Fig. 1*B*.

Finally, we tested HEK293 cells transfected with 3×FLAG-ZNF224 for specific interaction with the endogenous PRMT5 protein using a polyclonal anti-PRMT5 antibody. As shown in Fig. 1*C*, the specific interaction occurred only in HEK293 cell extracts immunoprecipitated with the FLAG antibody (*lane 2*), and not in the cells immunoprecipitated with control immunoglobulin (IgG, *lane 3*). Collectively, these results indicate a specific *in vivo* interaction between ZNF224 and PRMT5.

To determine whether these two proteins directly interact, we did an *in vitro* binding assay using recombinant GST fusion full-length ZNF224, GST-KRAB, and [^{35}S]Met-labeled PRMT5 produced by *in vitro* transcription/translation assay. After the GST-pulldown assay, the pelleted proteins were subjected to SDS-PAGE, and the dried gel was autoradiographed. As shown in Fig. 2*A*, PRMT5 was pulled down by GST-ZNF224 (*lane 3*), but not by GST alone (*lane 2*) or by GST-KRAB (*lane 4*), thus

0.1% Tween 20, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitors). The resin was washed three times with T&G buffer, boiled in Laemmli buffer, and analyzed by SDS-PAGE electrophoresis and subsequently detected by autoradiography.

Analysis of PRMT5 and Histone H4 Symmetric Dimethyl R3 Expression by Flow Cytometry—For detection of PRMT5 and H4R3 expression, ethanol-fixed HEK293 cells were washed with phosphate-buffered saline, permeabilized with Tween 20 and incubated for 60 min with rabbit polyclonal antibodies against PRMT5 (Upstate) or H4(Me_2)R3 (Active Motif) or rabbit IgG (Santa Cruz Biotechnology), as control. After washing, cells were incubated with fluorescein isothiocyanate-conjugated Goat Anti-Rabbit Ig secondary antibody (BD Pharmingen) for 60 min. Finally, cells were washed with phosphate-buffered saline and stained with propidium iodide overnight. Flow cytometric analysis was carried out by using the FACScan to Flow Cytometer (BD Biosciences).

RESULTS

Identification of Proteins Associated with ZNF224—Using FLAG antibody affinity chromatography and matrix-assisted laser desorption ionization analysis we identified the arginine methyltransferase PRMT5 as the 70-kDa ZNF224-associated component and, in addition, the 50-kDa MEP50 protein, a well

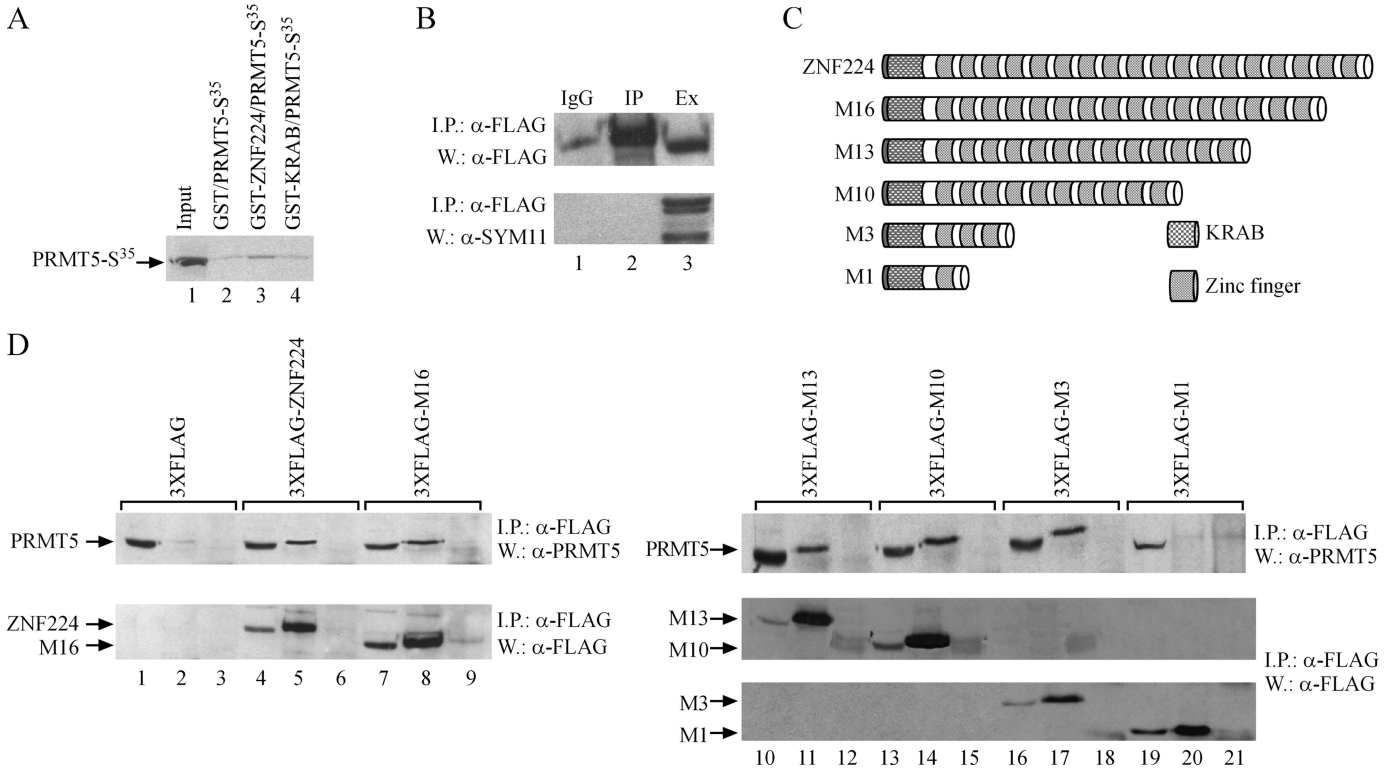

FIGURE 2. **PRMT5 interacts with ZNF224 *in vitro*.** *A*, [35S]Met-labeled PRMT5 was tested for direct interaction with GST-ZNF224 (*lane 3*), GST-KRAB (*lane 4*), and GST alone (*lane 2*). In *lane 1* was loaded 25% of input protein. *B*, ZNF224 exogenously expressed in HEK293 cells was immunoprecipitated with anti-FLAG. Western blot analysis was performed with anti-SYM11 antibody. *C*, schematic representation of the ZNF224 3′-deletion mutants. *D*, total protein lysates from HEK293 cells expressing 3×FLAG-ZNF224 or deletion mutants M16, M13, M10, M3, and M1 were immunoprecipitated with anti-FLAG (*lanes 5*, *8*, *11*, *14*, *17*, and *20*, respectively). Samples were analyzed by Western blot for expression of the FLAG tag epitope. The immunoprecipitate from HEK293 cells expressing empty p3x-FLAG vector (*lane 2*) was used as control. *Lanes 1*, *4*, *7*, *10*, *13*, *16*, and *19* show total protein lysates from transfected cells. *Lanes 3*, *6*, *9*, *12*, *15*, *18*, and *21* show the IgG immunoprecipitation, as control. Data shown were confirmed by three independent experiments.

ZNF224 Domain	Localization	Lenght	Zinc Finger Type	Identity with other ZNF Proteins
Zinc finger 1	176 – 198	23	C2H2-type	ZNF221 (78%) ; ZNF222 (91%) ZNF223 (86%) ; ZNF230 (91%) ZNF234 (91%) ; ZNF155 (81%)
Zinc finger 2	204 – 226	23	C2H2-type	ZNF221 (95%) ; ZNF222 (95%) ZNF223 (91%) ; ZNF225 (82%) ZNF234 (100%) ; ZNF155 (100%)
Zinc finger 3	232 – 254	23	C2H2-type	ZNF222 (73%) ; ZNF225 (78%) ZNF226 (78%) ; ZNF234 (86%) ZNF284 (69%) ; ZNF155 (91%)

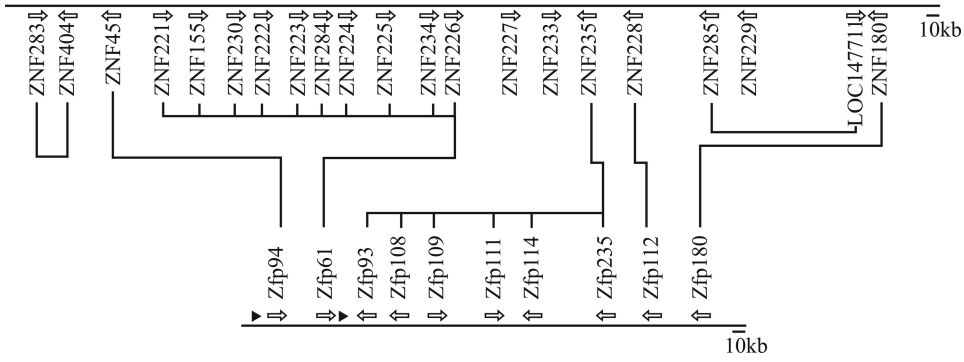

FIGURE 3. **ZNF224 zinc finger domains 1–3 are conserved within a subfamily of KRAB-ZNF proteins.** *Top panel*, *table* showing homology of ZNF224 zinc finger domains 1–3 with other ZNF proteins. *Bottom panel*, organization of predicted orthologs and paralogs in the human (*top*) and mouse (*bottom*) ZNF gene family region, according to Shannon *et al.* (18).

indicated that PRMT5 directly interacts with ZNF224 and that the KRAB domain is not sufficient for this interaction. The Coomassie Blue-stained gel showing levels of recombinant GST proteins used in the pulldown assay is reported in supplemental Fig. S1.

To determine if ZNF224 is a target of PRMT5 methylation, we immunoprecipitated HEK293 cells, transfected with 3×FLAG-ZNF224, with FLAG antibody. The immunoprecipitated ZNF224 was analyzed by Western blotting with an anti-SYM11 antibody, which specifically recognizes symmetrically dimethylated arginine. As shown in Fig. 2*B*, anti-SYM11 antibody did not detect dimethylated arginine in ZNF224 (*lane 2*, *lower panel*).

To identify the ZNF224 region involved in the interaction with PRMT5, a series of 3′-deletion mutants was produced and tested in immunoprecipitation assays. HEK293 cells were transfected with

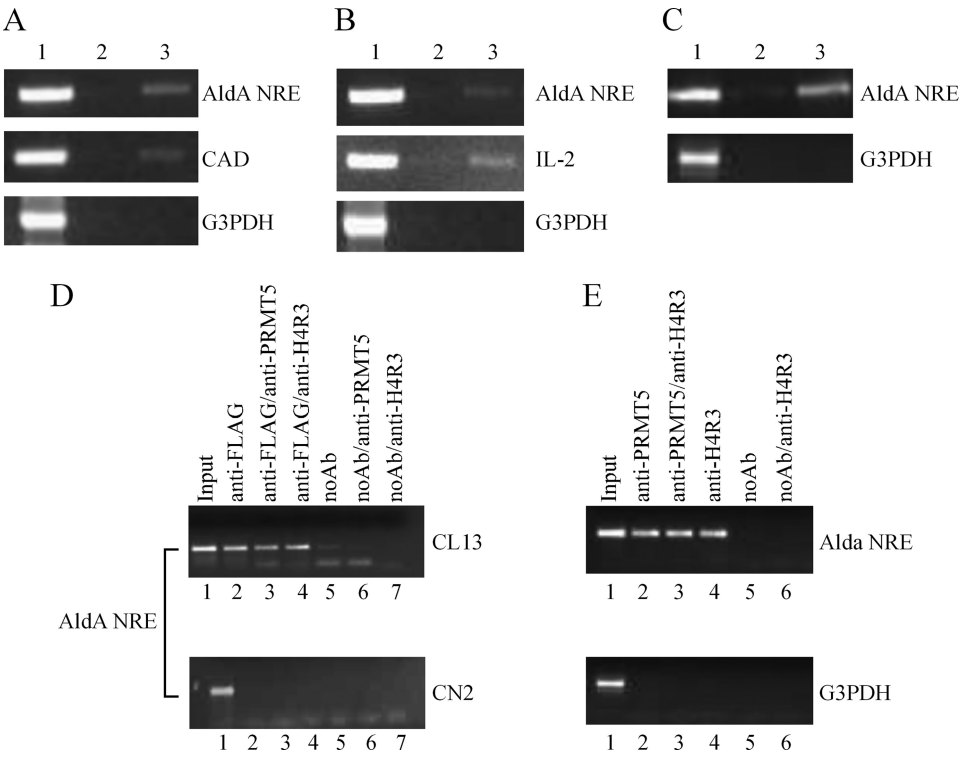

FIGURE 4. **PRMT5 is recruited to L-type aldolase A promoter.** ChIP assays were conducted using cross-linked chromatin from HEK293 cells with anti-PRMT5 (*panel A*), or anti-SYM11 (*panel B*), or anti H4R3 antibodies (*panel C*). The input chromatin (*lane 1*), the reaction mixture with chromatin but without antibody (*lane 2*) or the immunoprecipitated chromatin (*lane 3*) were PCR-amplified with specific primers, as indicated. Re-ChIP experiments were performed in HEK293 cells stably transfected with the expression vector encoding 3×FLAG-ZNF224 (*CL13*) or with the empty expression vector p3×FLAG, as negative control (*CN2*). A first immunoprecipitation was carried out with anti-FLAG antibody and a second precipitation with anti-PRMT5 (*panel D*, *lane 3*) or anti-H4R3 (*panel D*, *lane 4*). Precipitations without antibodies served as specificity controls (*panel D*, *lanes 5–7*). In *panel E* re-ChIP experiments were performed with a first immunoprecipitation with anti-PRMT5 (*lane 2*) and a second precipitation with anti-H4R3 (*lane 3*). *Lane 4* shows a ChIP assay with anti-H4R3. Precipitations without antibodies served as specificity controls (*lanes 5* and *6*). Samples were PCR-amplified with specific primers in the *upper* and *lower parts* of *panel E*, respectively, as indicated. Data shown were confirmed by three independent experiments.

the expression vector encoding for 3×FLAG-ZNF224 or expression vectors encoding for deletion mutants of ZNF224 containing 16, 13, 10, 3, and 1 zinc finger domains, respectively (M16, M13, M10, M3, and M1). Cell lysates were immunoprecipitated with FLAG antibody and subsequently immunoblotted with anti-PRMT5 antibody. As shown in Fig. 2*D*, the progressive deletion of the zinc finger domains from the COOH terminus of ZNF224 as far as the third zinc finger (M3) did not affect the interaction with PRMT5 (see *lanes 5*, *8*, *11*, *14*, and *17*, *upper panel*). Instead, the M1 clone, that includes only the KRAB domain plus the first zinc finger domain, was unable to bind PRMT5 (see *lane 20*, *upper panel*). This experiment indicates that the binding site for PRMT5 at least requires the second and third zinc finger domains of ZNF224. These domains are well conserved in some of the KRAB-ZFPs that are evolutionary related (see Fig. 3 and supplemental Table 1) (18). Based on this observation it is intriguing, indeed, to hypothesize that other KRAB-ZFPs may interact with PRMT5.

Recruitment of PRMT5 to the Aldolase A Promoter—In a previous report we found, using ChIP and co-transfection assays, that the ZNF224 repressor protein was recruited to the negative regulatory element AldA-NRE and repressed transcription of the aldolase A gene. We also demonstrated that ZNF224-mediated transcriptional repression requires the 45-amino acid long KRAB A domain and the specific interaction with the KAP-1 co-repressor molecule. Finally, we found that the selective inhibitor of histone deacetylases, trichostatin A, was able to remove the ZNF224-mediated repression (32).

Here, we used ChIP to investigate whether the ZNF224-associated PRMT5 arginine methyltransferase is directly recruited onto the promoter of aldolase A gene. Chromatin was prepared from HEK293 cells, and the immunoprecipitated samples were obtained using the anti-PRMT5 antibody (Fig. 4*A*). PCR analyses, performed using the oligonucleotides covering the AldA-NRE element, yielded the expected 300-bp product (*lane 3*, *upper panel*). Amplification of a specific fragment from the *cad* promoter, which is a well recognized PRMT5-regulated gene, confirmed that anti-PRMT5 antibody immunoprecipitated selectively its specific promoter targets (*lane 3*, *middle panel*) (11). No band was observed when a G3PDH fragment was amplified on the same chromatin samples immunoprecipitated with anti-PRMT5, thus demonstrating that PRMT5 does not recognize G3PDH as a target gene (*lane 3*, *lower panel*).

To determine whether any potential PRMT5 substrate is present *in vivo* on the L-type aldolase A promoter, we repeated our ChIP analyses with anti-SYM11 antibody. As shown in Fig. 4*B* (*lane 3*, *upper panel*), the anti-SYM11 antibody detected one or more symmetric dimethylation-containing proteins that were present on the L-type aldolase A promoter. Immunoprecipitation with anti-SYM11 antibody was confirmed by amplification of a specific fragment of the IL-2 promoter (*lane 3*, *middle panel*), to which methylated proteins are associated (14). No amplified product was detectable when specific GAPDH oligonucleotides were used on the same chromatin samples immunoprecipitated with anti-SYM11 (*lane 3*, *lower panel*).

Arginine 3 of histone H4 (H4R3) is a well known substrate for PRMT5. To investigate whether PRMT5 recruitment to the L-type aldolase A promoter correlates with methylation of H4R3, we performed ChIP experiments on HEK293 cells using an antibody against dimethylated histone H4R3. As shown in Fig. 4*C*, when anti-H4R3 antibodies were incubated with cross-linked chromatin from HEK293 cells, AldA-NRE was amplified (*lane 3*, *upper panel*). No amplified product was detectable

when specific GAPDH oligonucleotides were used on the same chromatin samples immunoprecipitated with anti-H4R3 antibodies (*lane 3*, *lower panel*).

To determine whether there was co-recruitment of ZNF224 and PRMT5 on the L-type aldolase A promoter, we performed sequential ChIP experiments using HEK293 cells stably transfected with the expression vector encoding 3×FLAG-ZNF224 (CL13 clone). Initial rounds of immunoprecipitation were performed with the anti-FLAG antibody, and then the immunoprecipitated chromatin was re-immunoprecipitated with anti-PRMT5 and anti-H4R3 antibodies. As shown in the *upper panel* of Fig. 4*D*, ZNF224 associated with PRMT5 on the L-type promoter (*lane 3*), but not in the negative control (*lane 6*), thus demonstrating that ZNF224 and the arginine methyltransferase PRMT5 may be present simultaneously on the L-type promoter region. Next, to verify the modification of the ZNF224-PRMT5-associated histone tail *in vivo*, we performed a re-ChIP assay with an antibody recognizing the methylated H4R3. A PCR product representing the L-type promoter fragment was obtained by re-immunoprecipitation in the presence of the anti-H4R3 antibody (*lane 4*) but not in absence of anti-FLAG antibody (*lane 7*).

This indicates that H4R3 is symmetrically dimethylated by PRMT5 *in vivo* on the nucleosomes surrounding the L-type promoter region. The same experiment was carried out on HEK293 cells stably transfected with the empty expression vector 3×FLAG, as control (CN2 clone) (Fig. 4*D*, *lower panel*).

This result was confirmed by a re-ChIP experiment with the PRMT5/H4R3 antibody combination (Fig. 4*E*, *upper panel*). Chromatin from HEK293 cells was first immunoprecipitated with PRMT5 antibody and then with H4R3 antibody. A PCR product corresponding to the amplified AldA-NRE fragment was obtained from immunoprecipitates with PRMT5 antibody (*lane 2*) and from re-ChIP with H4R3 antibody (*lane 3*). The *lower panel* of Fig. 4*E* shows the amplification of a G3PDH fragment on the same chromatin samples immunoprecipitated with the PRMT5/H4R3 antibody combination, as negative control. The results of the ChIP and re-ChIP experiments show that PRMT5 is recruited by ZNF224 to the L-type aldolase A promoter, where it induces arginine methylation of histone H4 on nucleosomes that surround the L-type promoter region. This suggests that PRMT5 is involved in the transcriptional control of aldolase A expression.

PRMT5 Knockdown Enhances L-type Aldolase A mRNA Expression—Because ChIP experiments demonstrated that PRMT5 was associated with the L-type aldolase A promoter *in vivo*, we tested whether this association was essential for aldolase repression. To examine the effect of PRMT5 on L-type aldolase A transcription, we knocked down the expression of PRMT5. HEK293 cells were transfected with a siRNA negative control and three different siRNAs against PRMT5, targeted to different sites in PRMT5 mRNA. We observed a similar ability of these siRNAs to decrease the expression of PRMT5 both at mRNA (Fig. 5*A*) and protein levels (Fig. 5*B*), 48 h after siRNA transfection. We then examined whether the knockdown of PRMT5 affected the expression of L-type aldolase A mRNA. As shown in Fig. 5*A*, the expression of L-type aldolase A mRNA increased significantly in the presence of PRMT5 siRNAs but not with an equivalent amount of the stealth RNA interference-negative control. These data reinforce the concept that PRMT5 is required to enable the ZNF224 transcriptional repressor complex to regulate aldolase A gene expression.

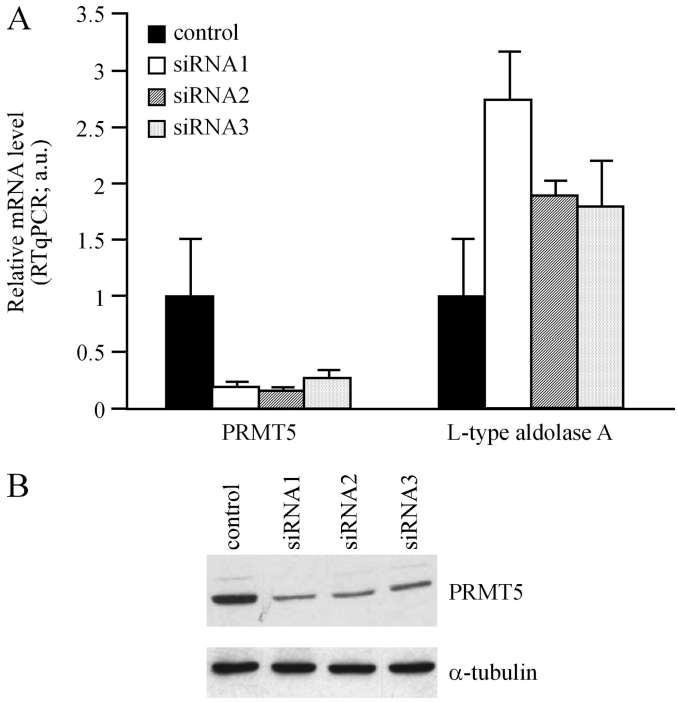

FIGURE 5. **PRMT5 knockdown affected the expression of L-type mRNA.** *A*, HEK293 cells were transfected with three different stealth RNA interference duplexes for PRMT5 (*siRNA1*, *siRNA2*, and *siRNA3*) and one stealth RNA interference-negative control duplex. After 48 h, the cells were lysed and the PRMT5 and L-type mRNA levels were evaluated by real-time PCR. *Error bars* indicate mean value ± S.D. of three independent experiments. *B*, protein extracts were analyzed by Western blot with anti-PRMT5 or anti-tubulin antibodies.

PRMT5 Association with Aldolase A Promoter during Different Phases of the Cell Cycle—Our previous data indicated that the periodic association of the transcriptional repressor complex to the AldA-NRE modulates L-type aldolase A gene expression during cell cycle and cell differentiation. In fact, we found that the repressor complex down-regulates the transcription of the L-type promoter in proliferating cell types, whereas the binding activity of the complex to the DNA is inhibited in growth-arrested cells, and this correlates with an increased L-type gene transcription (29, 30).

To verify whether PRMT5 is required for the ZNF224-mediated transcriptional repression, we examined the association of PRMT5 with the L-type promoter in cell cycle-arrested HEK293 cells by ChIP. Cells were arrested in G_0/G_1 and S phases using a thymidine block or in G_2/M phase by nocodazole treatment, and cell synchronization was monitored by flow cytometry of propidium iodide-stained cells (Fig. 6*A*). The modulation of aldolase A mRNA expression during the cell cycle was monitored using a real-time PCR assay (Fig. 6*B*). We measured the amount of the immunoprecipitated DNA fragment derived from the AldA-NRE region (and from a downstream region of the L-type promoter) *versus* the amount of the input DNA to determine the association of PRMT5 to L-type aldolase A promoter during the cell cycle. Promoter-bound

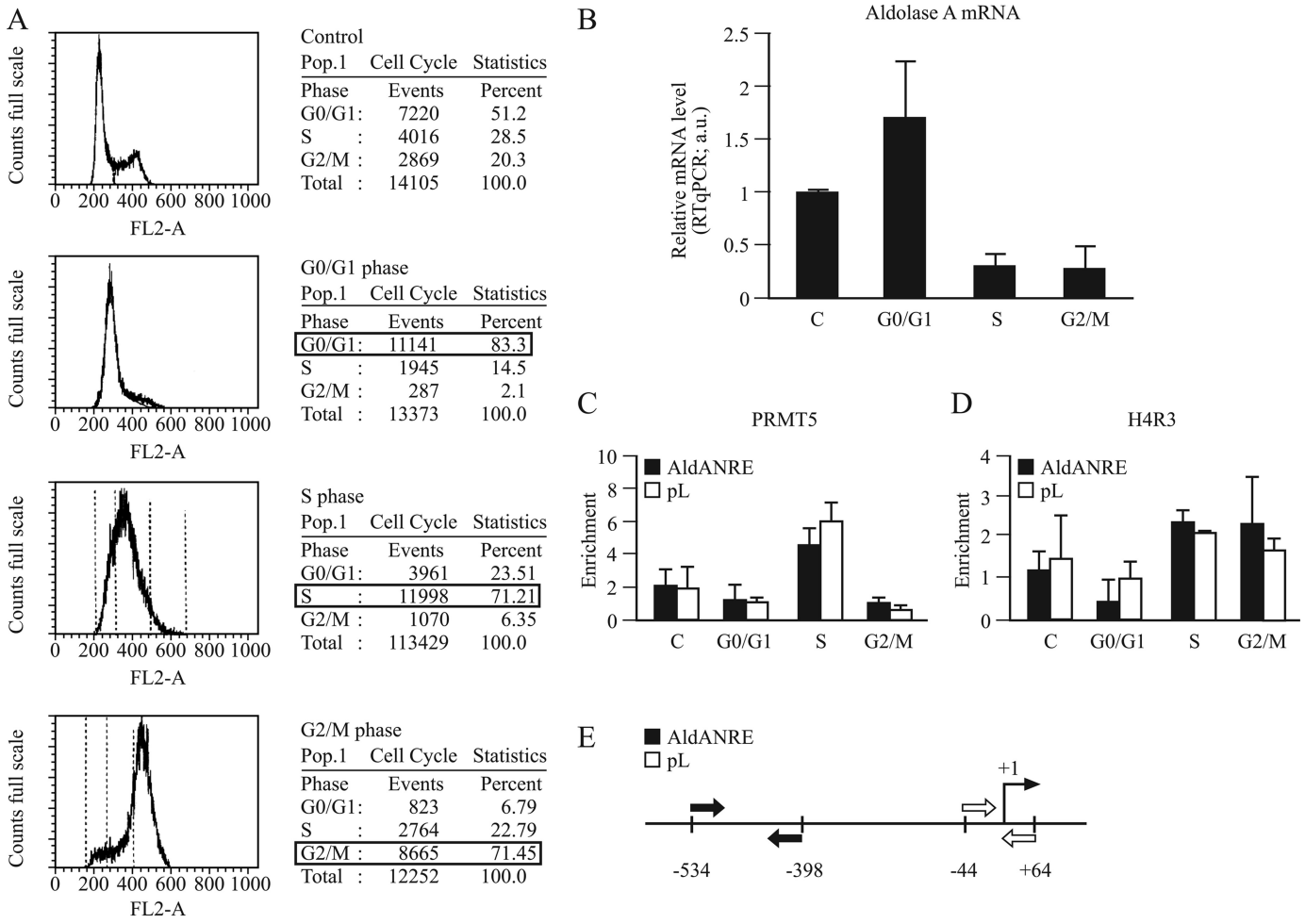

FIGURE 6. **Cell cycle-dependent recruitment of PRMT5 to the aldolase A promoter.** *A*, flow cytometry of cell cycle-arrested HEK293 cells. *B*, quantitative analysis of aldolase A L-type mRNA expression during the cell cycle. *Error bars* indicate mean value ± S.D. of three independent experiments. *C*, quantitative analysis by real-time PCR of PRMT5 binding to AldA-NRE and pL regions. *Error bars* indicate mean value ± S.D. of three independent experiments. *D*, quantitative analysis by real-time PCR of symmetric dimethylated arginine H4R3 during the cell cycle phases. *C* is a sample of proliferating cells. Data correspond to a single experiment representative of three independent experiments. *Error bars* indicate mean value ± S.D. of three independent experiments. *E*, schematic representation of the pL promoter region of aldolase A gene.

PRMT5 decreased when the cells were arrested in the G_0 phase, and the expression of L-type mRNA increased. On the contrary, PRMT5 was bound to chromatin when synchronized cells entered S phase. During this time, the expression of L-type mRNA was reduced (Fig. 6, *B* and *C*). Therefore, the recruitment of PRMT5 to the aldolase A promoter during G_0/G_1 and S phases of the cell cycle well correlates with L-type mRNA expression. Instead, in cells arrested at G_2/M the binding of PRMT5 to chromatin and the L-type mRNA expression were both significantly lower. The methylation of the nucleosomes surrounding the L-type promoter region on H4R3 was increased in cells at phases S and G_2/M, when the expression of L-type mRNA was low (see Fig. 6, *B* and *D*). Therefore, it appears that in G_2/M phase, notwithstanding the absence of PRMT5 on the repression complex, H4R3 is still methylated and able to prevent aldolase A gene transcription.

Finally, we examined the level of PRMT5 and global methylation of histone H4R3 during the cell cycle by flow cytometric analysis (Fig. 7). We observed a progressive increase of PRMT5 expression and of symmetric histone H4R3 methylation when HEK293 cells pass through S and G_2/M phases of the cell cycle. This result indicates that changes of the H4R3 methylation status may take part to the epigenetic control of cell cycle. Altogether, these experiments suggest that the cell cycle-dependent recruitment of the PRMT5-ZNF224 repression complex on the aldolase A promoter determines a regulated L-type mRNA expression and is dependent on epigenetic modifications of chromatin.

DISCUSSION

The aim of our study was to gain novel insight into the molecular mechanisms underlying KRAB-ZFP-mediated repression by analyzing the function of ZNF224 in aldolase A gene expression (31). In fact, although many KRAB-ZFPs have been identified, the target genes of only a few of them have been reported, and the transcriptional repression mechanisms are still far from being clarified. Many results were obtained in *in vitro* experiments, without taking account of the chromatin organization. Our physiological model of KRAB regulation of gene expression, based on cell cycle modulation by the ZNF224-AldA-NRE interaction, allows a better definition of a general mechanism of gene repression.

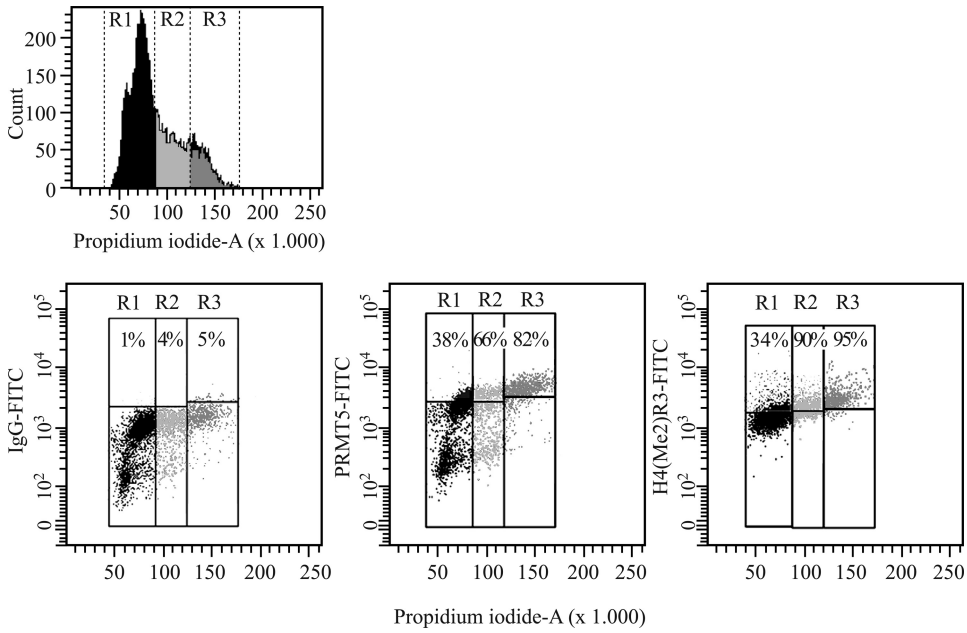

FIGURE 7. **Expression of PRMT5 and H4 symmetric dimethyl R3 during the cell cycle.** *Top panel*, flow cytometric histogram of DNA content of HEK293 cells, stained with propidium iodide. Three gates were placed according to G_0/G_1 (*R1*), S (*R2*), and G_2/M (*R3*) cell cycle phases. *Bottom panels*, flow cytometric histograms of PRMT5 and H4(Me_2)R3 expression in HEK293 cells. Cells, stained with propidium iodide and the indicated antibodies, were acquired in flow cytometry. The percentage of positive cells in each gate was calculated relative to the isotype control antibody (IgG-FITC). Data shown are representative of three independent experiments.

Using an *in vitro* and *in vivo* approach (transient transfection and chromatin immunoprecipitation assays), we previously demonstrated that ZNF224 represses aldolase A gene transcription through interaction with KAP-1 co-repressor protein (32). Several reports indicate the co-repressor KAP-1 is a scaffold protein that interacts with a network of histone-modifying activities (SETDB1, HP1, and Mi-2α) to address the transcriptional repression complex on the promoter region of target genes (25–27). In line with these reports, we demonstrated that ZNF224-mediated repression requires the specific ZNF224KRAB-KAP-1 interaction and the recruitment of enzymatic activities, *i.e.* histone deacetylase 1, to inhibit the human aldolase A gene transcription (32).

In this study, we asked whether other regulatory proteins are necessary to form the ZNF224-mediated repression complex. To this aim we performed co-immunoprecipitation experiments and demonstrated that PRMT5, a type II protein arginine methyltransferase, is physically associated with the KRAB-ZFP ZNF224, the aldolase A gene repressor (Figs. 1 and 2). Moreover, ChIP assays showed that PRMT5 is recruited to the L-type aldolase A promoter and that *in vivo* methylation of arginine 3 of histone H4 occurs when PRMT5 occupies the negative regulatory element of aldolase A gene (AldA-NRE) (Fig. 4).

PRMT5 has been shown to regulate various cytoplasmic processes, including biogenesis of small nuclear ribonucleoprotein particles by methylating Sm proteins (9, 10). Recently, the nuclear function of PRMT5 has come under scrutiny. PRMT5 has been demonstrated to methylate histones H3 and H4, transcription factors, and co-regulators, thus leading to the fine regulation of gene expression. PRMT5, for example, plays a role in the expression of IL-2 by recruiting methylarginine-specific protein(s) to cytokine promoter regions (14). More recently, a role for PRMT5 has been highlighted in the regulation of cell growth and proliferation, by expression control of genes involved in tumor suppression and cell cycle (11–13). Moreover, it has recently been demonstrated that p53 is a substrate for PRMT5 methylation (35).

This prompted us to verify the recruitment of PRMT5 to the promoter regions of genes encoding proteins involved in cell cycle regulation using the cell cycle-dependent modulation of L-type aldolase A promoter activity as an experimental tool (29, 30). Our findings indicate that PRMT5 is involved in the formation of the complex that negatively regulates aldolase A gene transcription during the cell cycle (Fig. 6). Therefore, based also on our finding of changes in PRMT5 expression and symmetric H4R3 dimethylation during the cell cycle (Fig. 7), we speculate that PRMT5-mediated methylation of histones is required to elicit repression of genes whose function is correlated to the alternating phases of the cell cycle.

Histone tail modifications represent transient marks modulating chromatin structure, thus allowing orderly progression of cell cycle. For a clearer understanding of the molecular basis of this epigenetic mechanism, it is also required to elucidate the role played by different histone modifications on the modulation of chromatin structure. Moreover, our results represent the first experimental evidence that the association between a KRAB-ZFP and the arginine methyltransferase PRMT5 is necessary to mediate gene repression of its target gene (Fig. 4).

It is well known that KRAB-ZFPs recruit the co-repressor KAP-1 on the promoter region of their target genes via the KRAB domain (23, 24). KAP-1 is generally considered a platform protein that coordinates the assembly of histone deacetylase, histone methylase and deposition of HP1 proteins to silence gene expression by forming a facultative heterochromatin environment (25–28). KRAB-mediated transcriptional repression depends on KAP-1 and on the network of proteins binding to KAP-1. Recently, Sripathy *et al.* (36) demonstrated that KAP-1 co-repressor functions to coordinate the assembly of the *de novo* HP1-demarcated microenvironment of heterochromatin required for KRAB-ZFP-mediated transcriptional repression. Another report confirmed that methylation of specific lysine residues on histones surrounding a promoter region is a marker of a KAP-1-mediated transcriptional repression complex on that gene (37).

Our experiments demonstrate that a direct interaction between the arginine methyltransferase PRMT5 and ZNF224 is

required for repression of aldolase A gene transcription. Although we cannot exclude that PRMT5 interacts with other proteins of the ZNF224 transcriptional repression complex, we suggest that methylation of H4R3 by PRMT5 is involved in the modulation of aldolase A gene transcription (Figs. 4–6). The identification of the ZNF224-PRMT5 interaction region (Fig. 2) and its high homology within a subset of KRAB-ZFP belonging to the ZNF224 family (Fig. 3) allow us to speculate that PRMT5 is a key mediator for the regulation of KRAB-ZFP-mediated repression and provide new information on chromatin modifications involved in gene repression, opening the way to further investigations.

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